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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the _____ separately numbered sheets attached hereto				
TITLE OF THE INVENTION (280 characters max)				
IN VIVO METHODS FOR VALIDATING THE ROLE OF A TUMOR MAINTENANCE GENE				
Direct all correspondence to: CORRESPONDENCE ADDRESS				
<input checked="" type="checkbox"/> Customer Number	1473		Place Customer Number Bar Code Label here	
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ENCLOSED APPLICATION PARTS (check all that apply)				
<input checked="" type="checkbox"/> Specification	Number of Pages	14	<input type="checkbox"/> CD(s), Number	
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<input checked="" type="checkbox"/> Application Data Sheet	See 37 CFR 1.76			
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT				
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE AMOUNT (\$)
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees				
<input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:	06-1075			\$80.00
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.				
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.				
<input checked="" type="checkbox"/> No.				
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are:				

Respectfully submitted,

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Date

December 24, 2002

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Docket No.

GEN/005 PROV.

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Hon. Commissioner for Patents, Washington, D.C. 20231.

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GEN-005 PROV.
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PROVISIONAL PATENT APPLICATION

Applicants: : Rónán C. O'Hagan et al.

For : IN VIVO METHODS FOR VALIDATING THE ROLE
OF A TUMOR MAINTENANCE GENEBOX PROVISIONAL PATENT APPLICATION
HON. COMMISSIONER FOR PATENTS
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Arlington, Virginia 22202New York, New York 10020
December 24, 2002EXPRESS MAIL CERTIFICATIONExpress Mail Label No.: EV133111761US
Date of Deposit: December 24, 2002

I hereby certify that this certification and the following papers and fees:

1. Provisional Application Cover Sheet (in duplicate);
2. Specification
3. PrintEFS Data Sheet
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are being deposited with the United States Postal Service "Express Mail Post Office to Address" service under 37 C.F.R. § 1.10 on the date indicated above and are addressed to BOX PROVISIONAL PATENT APPLICATION, Commissioner for Patents, P.O. Box 2327, Arlington, VA 22202.

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APPLICATION INFORMATION

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Title Line Two:: OF A TUMOR MAINTENANCE GENE
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GEN/005 PROV

**IN VIVO METHODS FOR VALIDATING
THE ROLE OF A TUMOR MAINTENANCE GENE**

BACKGROUND OF THE INVENTION

5 The impact of cancer on our society cannot be understated. Despite the
long history of clinical and research efforts directed towards understanding cancer,
surprisingly little is known about the genetic lesions responsible for its genesis,
progression, and clinical behavior. For example, in the case of melanoma, although
many genes have been implicated in the genesis of this disease, only the INK4a, RAS
and BRAF genes have been shown to be true etiologic lesions in a formal genetic
10 sense (Chin et al., Genes Devel. 11:2822-34 (1997); Davies et al., Nature 417:949-54
(2002)). Moreover, advanced malignancy represents the phenotypic endpoint of many
successive genetic lesions that affect many oncogene and tumor suppressor gene
pathways. Lesions that lead to such a condition may therefore differ from those
required to maintain it. Both types of lesions represent rational therapeutic targets in
15 the treatment of cancer. Thus, there is a need for identifying genes involved in tumor
maintenance and for rapidly confirming the tumor maintenance functions of these
genes.

SUMMARY OF THE INVENTION

20 This invention provides *in vivo* methods for confirming the tumor
maintenance function of a candidate gene. In these methods, a mammalian host cell is

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generated, which comprises (1) an oncogene expression construct (e.g., *H-ras*^{V12G}), (2) a genetic mutation or an RNA reagent (e.g., an RNA interference molecule, an antisense RNA, or a ribozyme) that causes reduced expression or activity of a tumor suppressor gene (e.g., *Ink4a/arf*), and (3) a coding sequence for an RNA interference molecule against said candidate gene, said coding sequence linked operably to an inducible promoter. This mammalian host cell or progeny thereof is introduced into an animal of a desired developmental stage (e.g., a blastocyst, or an adult animal). Due to the oncogene expression and the cancer-prone genetic mutation or RNA reagent, the host cell or progeny thereof may develop into a tumor *de novo* in the animal. Once a tumor is established, expression of the interfering RNA is induced, wherein subsequent regression of the tumor indicates that said candidate gene is essential for tumor maintenance. As used herein, tumor maintenance functions include those required to sustain tumor size, to maintain tumor viability including preventing tumor cell death and differentiation, and to facilitate tumor growth including angiogenesis and metastasis. Tumor regression is indicated by, e.g., apoptosis of tumor cells, reduction in tumor size, collapse of tumor vasculature, and reversion of tumorigenic phenotype of tumor cells

Once the tumor maintenance role of a candidate gene is confirmed, the animal can further be used to determine the extent of inhibition of the gene required to inhibit or reverse tumor growth without causing general side effects. This is made possible by the inducibility of the RNAi molecules in nontumor tissues in the animal. Different extents of inhibition of the gene can be effected by, e.g., using different RNAi molecules and/or inducing different levels of the molecules. Thus, the animal and method of this invention facilitate the definition of therapeutic windows for therapies that purport to inhibit the expression and/or activity of the gene.

Other features and advantages of the invention will be apparent from the following detailed description, and from the drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a drawing illustrating a single-vector system for validating the tumor maintenance function of a candidate gene. "T" denotes thymidine. "GPC-RNAi" denotes a coding sequence for an RNA that interferes with the expression of a candidate (GPC) gene. "ts-RNAi" denotes the coding sequence for an RNA that interferes with the expression of a tumor suppressor gene. "U6" denotes a U6 promoter. "LacO" denotes a Lac operator sequence. "LacI" denotes the coding sequence for a Lac repressor. "IRES" denotes an internal ribosome entry site. "YFP" denotes the coding sequence for a yellow fluorescent protein. "GW" means a "gateway" cassette that can be readily replaced by another GPC-RNAi coding unit.

Fig. 2 is a drawing illustrating a two-vector system for validating the tumor maintenance function of a GPC gene. In this system, ts-RNAi, LacI and YFP reside in one vector, while GPC-RNAi and an oncogene reside in the other vector.

Fig. 3 is a drawing illustrating another two-vector system for validating the tumor maintenance function of a GPC gene. "rtTA" denotes the coding sequence for a reverse tetracycline transactivator. "TetOp" denotes a tetracycline operator sequence. In this system, rtTA, LacI and YFP reside in one vector, while ts-RNAi, GPC-RNAi and an oncogene reside in the other vector.

Fig. 4 is a drawing illustrating a single-vector system that utilizes (1) zygotes from a transgenic animal having a cancer-prone genetic mutation such as a null mutation in *Ink4a/arf* and a transgene for a dominant acting *ras*, or (2) embryonic stem (ES) cells having such a genetic mutation.

DETAILED DESCRIPTION OF THE INVENTION

To confirm the role of a candidate gene in tumor maintenance, it is important to prove that the gene is essential for maintenance of tumors that arise *de novo*. To do this, one can inhibit the activities of the candidate gene in an established tumor and determine whether the inhibition causes tumor regression. Inhibition of a candidate gene's activity can be accomplished by, e.g., regulated gene expression knockdown by RNA interference (RNAi), or conditional gene knockout.

I. GENE EXPRESSION KNOCKDOWN BY RNA INTERFERENCE

A. Interfering RNAs

A double-stranded RNA (dsRNA) that interferes with (i.e., inhibits or "knocks down") the expression of a candidate tumor maintenance gene can be expressed in an established tumor to determine whether this interference causes tumor regression. The dsRNA is homologous to the candidate gene and causes degradation of mRNAs of the candidate gene. The mediators of the degradation are 21- to 23-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from the longer dsRNAs (including hairpin RNAs). Molecules of siRNA typically have 2- to 3-nucleotide 3' overhanging ends resembling the RNAse III processing products of long dsRNAs that normally initiate RNAi. When introduced into a cell, they assemble with an endonuclease complex (RNA-induced silencing complex), which then guides target mRNA cleavage. As a consequence of degradation of the targeted mRNA, cells with a specific phenotype of the suppression of the corresponding protein product are obtained (e.g., reduction of tumor size, metastasis, angiogenesis, and growth rates). The small size of siRNAs, compared with traditional antisense molecules, prevents activation of the dsRNA-inducible interferon system present in mammalian cells. This helps avoid the nonspecific phenotypes normally produced by dsRNA larger than 30 base pairs in somatic cells. See, e.g., Elbashir et al., *Methods* 26:199-213 (2002); McManus and Sharp, *Nature Reviews* 3:737-747 (2002); Hannon, *Nature* 418:244-251 (2002); Brummelkamp et al., *Science* 296:550-553 (2002); Tuschl, *Nature Biotechnology* 20:446-448 (2002); U.S. Application US2002/0086356 A1; WO 99/32619; WO 01/36646; and WO 01/68836.

Double-stranded RNAs that interfere with expression of a target gene (e.g., for this invention, a candidate tumor maintenance gene or a tumor suppressor gene) can be designed with a number of software programs. An exemplary program is the OligoEngine siRNA design tool available at <http://www.oligoengine.com>. Preferred siRNAs of this invention range about 19-29 basepairs in length for the double-stranded portion. In some embodiments, the siRNAs are hairpin RNAs having an about 19-29 bp stem and an about 4-34 nucleotide loop. Preferred siRNAs are

- 5 -

highly specific for a region of the target gene and may comprise any 19-29 bp fragment of a target gene mRNA that has at least one (e.g., at least two or three) bp mismatch with a nontarget gene-related sequence. In some embodiments, the preferred siRNAs do not bind to RNAs having more than 3 mismatches with the target region.

B. Vectors for Expressing Interfering RNAs

Intracellular transcription of dsRNAs can be achieved by cloning the dsRNA-encoding sequences into vectors containing RNA polymerase III (Pol III) transcription units, which normally encode the small nuclear RNA U6 or the human RNAse P RNA H1, or RNA polymerase I (Pol I) or II (Pol II) transcription units (e.g., units containing a CMV promoter). Two approaches can be used for expressing dsRNA: (1) sense and antisense strands constituting the dsRNA duplex are transcribed by individual promoters; or (2) dsRNAs are expressed as fold-back stem-loop structures (hairpins) that give rise to dsRNAs after intracellular processing. An exemplary U6 transcription unit is as follows:

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GATTTAGGTGACACTATAGAATACAAGCTTGGCTGCAGGTCGACGGATCC
CCCCGAGTCCAACACCCGTGGGAATCCCATGGGCACCATGGCCCCCTCGCT
CCAAAAATGCTTTCGCGTCTCGCAGACACTGCTCGGTAGTTTCGGGGATC
AGCGTTTGAAGAGCCCGCTCTGAACCCTCCGCGCCGCCCCGGCCCC
20 AGTGAAAGACGCGCAGGCAAAACGCACCACGTGACGGAGCGTGACCGCG
CGCCGAGCGCGCGCCAAGGTGCGGCAGGAAGAGGGCCTATTTCCCATGAT
TCCTTCATATTGTCATATACGATACAAGGCTGTTAGAGAGATAATTAGAA
TTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGA
AAGTAATAATTTCTTGGGTAGTTTGCAGTTTTTAAAATTATGTTTTTAAA
25 TGGACTATCATATGCTTACCGTAACTTGAAAGTATTTTCGATTTCTTGGCT
TATATATCTTGTGGAAAGGACGAAACACCCGTGCTCGCTTCGGCAGCACA
TATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCCTGCGCAA
GGATGACACGCAAATTCGTGAAGCGTTCCATATTTTACATCAGGTTGTT
TTTCTGTTTTTACATCAGGTTGTTTTCTGTTTGGTTTTTTTTTTTACACC
30 ACGTTTATACGCCGGTGCACGGTTTACCA (SEQ ID NO:1)

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In this sequence, the U6-coding sequence is underlined, with the transcription initiation site GTG double-underlined. The promoter region spans from the first nucleotide to the nucleotide immediately preceding the GTG. The TATA box in the

promoter region is boxed. To construct an RNAi vector, the U6-coding sequence can be replaced in part or in its entirety by a sequence encoding an interfering dsRNA.

Inducible transcription-regulatory elements can also be inserted into the promoter region for controlled expression of the dsRNAs. Exemplary inducible
5 systems are the Lox-Cre systems; the tetracycline operator/repressor systems and Lac operator/repressor systems for Pol III; and the tetracycline transactivator systems, reverse tetracycline transactivator systems, ecdysone systems, and methallothionine systems for Pol II.

Various vectors can be used for the dsRNA expression. These vectors
10 can be based on plasmids or viruses such as retroviruses, adenoviruses, and lentiviruses. The RNAi vectors can be introduced into zygotes, ES cells, tissue-specific stem cells, organ explants or organs *in situ* as required, via a variety of methods, including but not limited to, liposome fusion (transposomes), routine nucleic acid transfection methods such as electroporation, calcium phosphate precipitation,
15 and microinjection, and infection by viral vectors.

C. Exemplary Lentiviral Vector Systems

In some embodiments, lentiviral vectors are used because they can infect dividing as well as nondividing cells and be stably integrated into the genome of infected cells. In these embodiments, an oncogene, a ts-RNAi, and an inducible GPC-
20 RNAi are cloned into one or two lentiviral vectors (see, e.g., Figs. 1-4). The lentiviral vectors are then used to infect a mammalian host cell (e.g., a murine cell or a human cell). Expression of the oncogene and inhibition of the tumor suppressor (ts) gene via RNA interference will transform the host cell into a tumor cell. If the candidate GPC gene contributes to tumor maintenance, induced expression of its interfering RNA will
25 lead to reversion of the tumor phenotype of the host cell.

One significant advantage of these vector systems is that each transformed host cell necessarily contains an inducible GPC-RNAi sequence (*infra*). Thus, tumor maintenance functions of the candidate GPC gene can be directly studied in the transformed cells, without the uncertainty as to whether or not any change in the

tumor status of a given cell is caused by the GPC-RNAi. Further, tumor regression caused by the GPC-RNAi will not be obscured by overgrowth of tumor cells not containing the GPC-RNAi. Also, the use of a Gateway (GW) cassette in the lentiviral vectors allows easy introduction of different GPC-RNAi coding sequences. Thus, these vector systems result in faster tumorigenesis as well as faster and more reliable validation of a candidate tumor maintenance gene.

The following describes several examples of the lentiviral vector systems. These examples are intended to illustrate the methods and materials of the present invention. Suitable modifications and adaptations of the described conditions and parameters normally encountered in the art which are obvious to those skilled in the art are within the spirit and scope of the present invention.

1. First Example

Fig. 1 illustrates this example. In this example, a single lentiviral vector is generated, which contains (1) a Gateway cassette containing a U6-based transcription unit for expressing a GPC-RNAi sequence, where a Lac operator sequence is inserted into the U6 promoter region; (2) a U6-based transcription unit for expressing a ts-RNAi sequence; (3) the respective coding sequences for a Lac repressor and a fluorescent protein (or any other protein designed to facilitate imaging of the infected cell), the two coding sequences separated by an IRES and both under the transcriptional control of a constitutive promoter; and (4) an oncogene under the transcriptional control of a constitutive promoter. In part (3) above, the Lac repressor coding sequence may be optionally modified to adapt to the codon usage by mammalian transcriptional systems.

Examples of ts genes used in part (2) are Ink4a/arf, pten, rb, and p53. Examples of fluorescent proteins used in part (3) are green fluorescent proteins, blue fluorescent proteins, yellow fluorescent proteins, and luciferases. Examples of oncogenes used in part (4) are dominant acting forms of H-ras, K-ras, N-ras, c-myc, n-myc, EGFR, MDM2, BDNF, her2/neu/erb-B2, TGF- β , RhoC, VEGF-C, AKT, abl, src, raf, fos, and β -catenin.

Examples of constitutive promoters used in parts (3) and (4) are CMV promoters, EF1 α , retroviral LTRs, and SV40 early regions. Constitutive promoters for driving expression the oncogene in part (4) can also include tissue-specific promoters, for example: a tyrosinase promoter or a TRP2 promoter in the case of melanoma cells and melanocytes; an MMTV or WAP promoter in the case of breast cells and/or cancers; a Villin or FABP promoter in the case of intestinal cells and/or cancers; a RIP promoter in the case of pancreatic beta cells; a Keratin promoter in the case of keratinocytes; a Probasin promoter in the case of prostatic epithelium; a Nestin or GFAP promoter in the case of CNS cells and/or cancers; a Tyrosine Hydroxylase, S100 promoter or neurofilament promoter in the case of neurons; the pancreas-specific promoter described in Edlund et al. Science 230:912-916 (1985); a Clara cell secretory protein promoter in the case of lung cancer; and an Alpha myosin promoter in the case of cardiac cells. Constitutive promoters for driving expression of the oncogene in part (4) can also be developmentally regulated promoters, which include, without limitation, the murine hox promoters (Kessel and Gruss, Science 249:374-379 (1990)) and the α -fetoprotein promoter (Campes and Tilghman, Genes Dev. 3:537-546 (1989)).

This lentiviral vector can be used to infect a zygote, an ES cell or a tissue-specific stem cell. The infected zygote can be developed into a transgenic animal. The infected embryonic or tissue-specific stem cell can be introduced into a host animal at any developmental stage to create a transgenic or chimeric animal harboring the infected cell and/or progeny of the infected cell. The constitutively expressed fluorescent protein is used to confirm infection and to follow tumor formation and regression.

2. Second Example

Fig. 2 illustrates this example. In this example, two lentiviral vectors are used to co-infect a host cell. The first vector contains (1) a U6-based transcription unit for expressing a ts-RNAi sequence; and (2) the respective coding sequences for a Lac repressor and a fluorescent protein, the two coding sequences separated by an

IRES and both under the transcriptional control of a constitutive promoter. The second vector contains (1) a Gateway cassette containing a U6-based transcription unit for expressing a GPC-RNAi sequence, where a Lac operator sequence is inserted into the U6 promoter region; and (2) an oncogene under the transcriptional control of a constitutive promoter. These two vectors can be used to co-infect host cells to create animal cancer models of any genotype at any developmental stage.

One characteristic of this two-vector system is the placement of the primary oncogenic elements, i.e., the ts-RNAi and the oncogene, on separate viruses. Consequently, tumor formation indicates the presence of both vectors in a host cell, because tumor transformation of the host cell depends on both oncogenic elements. Tumor formation will also indicate that other essential components of both vectors, including GPC-RNAi, are also in this host cell. Further, the two vector system improves bio-safety.

3. Third Example

Fig. 3 illustrates this example. In this example, two lentiviral vectors are used to co-infect a host cell. The first vector contains (1) a rtTA-coding sequence under the control of a constitute promoter; and (2) the respective coding sequences for a Lac repressor and a fluorescent protein, the two coding sequences separated by an IRES and both under the transcriptional control of a constitutive promoter. The second vector contains (1) a U6-based transcription unit for expressing a ts-RNAi sequence; (2) a Gateway cassette containing a U6-based transcription unit for expressing a GPC-RNAi sequence, where a Lac operator sequence is inserted into the U6 promoter region; and (3) an oncogene under the transcriptional control of the rtTA and tetracycline. These two vectors can be used to co-infect host cells to create animal cancer models of any genotype at any developmental stage.

Due to the inducible control of oncogene expression, this system further improves bio-safety. It also limits effects of oncogene expression to tumorigenesis; that is, the mere presence of an uninduced oncogene in the genome will not interfere with normal tissue development.

In lieu of the rtTA inducible system illustrated here, the expression of oncogene can also be controlled by any of the Lox-Cre or Pol II inducible systems described in Section B, *supra*. Further, instead of inducible promoters, the activity of the oncogene can also be inducibly switched on or off by fusing the oncogene protein to, e.g., an estrogen receptor polypeptide sequence, where administration of estrogen or an estrogen analog (e.g., hydroxytamoxifen) will allow the correct folding of the oncogene polypeptide into a functional protein.

4. Fourth Example

Fig. 4 illustrates this example. In this example, a single lentiviral vector is used to infect zygotes derived from tumor-prone animals, such as animals having an activating mutation in an oncogene and/or an inactivating mutation in a tumor suppressor gene. Examples of such animals include those described in WO 01/09308; Chin et al., Genes Devel. 11:2822-34 (1997); Leder U.S. Patent 4,736,866; and Beach U.S. Patent 5,919,997. The single vector contains (1) a Gateway cassette containing a U6-based transcription unit for expressing a GPC-RNAi sequence, where a Lac operator sequence is inserted into the U6 promoter region; and (2) the respective coding sequences for a Lac repressor and a fluorescent protein, the two coding sequences separated by an IRES and both under the transcriptional control of a constitutive promoter. The infected zygote can then be developed into a transgenic animal. This vector system ensures that the inducible GPC-RNAi coding sequence is contained within every cell of a tumor developed in the transgenic animal.

Alternatively, this vector is used to infect ES cell lines harboring an activating mutation in an oncogene and/or an inactivating mutation in a tumor suppressor gene. The ES cell lines can be derived, e.g., from the tumor-prone animals described above. The infected ES cells are then used to generate chimeric animals containing the lentiviral vector. The infected ES cells can also be used with tetraploid blastocysts to generate transgenic animals whose genome has an integrated lentiviral vector. Tumors originating from the ES cells are then induced in the chimeric or transgenic animals. Next, GPC-RNAi expression is induced in the established tumors.

The concomitant induction of GPC-RNAi in normal tissues of the animal enables one to assess toxic side effects of interference with the target gene.

5. Fifth Example

To improve the bio-safety of the above-described lentiviral vectors, one
5 can use engineered lentiviral vectors that can infect only nonhuman cells. For
instance, one can breed animals transgenic for the avian leucosis-sarcoma virus
receptor (ALSV) with the aforementioned tumor-prone animals to generate a
transgenic animal containing the tumor-prone genetic mutation as well as expressing
the ALSV. ALSV-A pseudotyped versions of the above-described lentiviral vectors
10 are then used to infect cells derived from this transgenic animal (e.g., zygotes, ES
cells, tissue-specific stem cells, etc.). The pseudotyped vectors can infect only cells
expressing the ALSV receptor. Thus, the vectors are not hazardous to humans.
Further, in lieu of ALSV-A pseudotyped lentiviral vectors, RCAS avian retroviral
vectors can also be used.

15 D. Establishment of Therapeutic Windows

The above-described RNAi systems will allow studies of tumor
regression as well as toxicity of inhibiting the candidate gene's expression or activity
in nontumor tissues in an animal. In some embodiments, zygotes infected by the
above lentiviral vectors or vector sets are developed into transgenic animals of desired
20 developmental stages (including embryonic stages). Subsequent to *de novo* tumor
formation in these animals, the GPC-RNAi expression is induced. One can then not
only observe the effects of the interfering RNA on the maintenance of a tumor that
arises *de novo* in the animal, but can also observe any undesired effects of the
interfering RNA on normal tissues of the animal. Further, by observing tumor
25 regression and concurrent toxicity caused by the RNAi, one can establish a window of
therapeutically effective amounts of an RNAi molecule, and thus the therapeutically
effective extent of inhibition of the target gene, that causes tumor regression without

general toxicity in the animal. This approach is particularly helpful if the animal is a nonhuman primate.

II. CONDITIONAL GENE KNOCKOUT

To knockout a candidate gene in a controlled manner, one can generate
5 animals or cells whose genome contains a pair of Lox sites flanking the candidate
gene or an essential region of the gene (e.g., a promoter region, or a region encoding a
domain essential for the gene product's activity). The genome is also engineered to
contain a Cre-encoding sequence under the control of an inducible promoter, a tissue-
specific promoter, or a developmental stage-specific promoter, such as those described
10 above. As a result, the candidate gene is knocked out only at a time and a place
dictated by the activity of the Cre promoter.

The animals or cells so generated are useful for evaluating the
tumorigenesis and tumor maintenance roles of a candidate gene by knocking out the
gene before and after tumor formation, respectively. They are also useful for
15 assessing the toxic effects of knocking out the candidate gene. For example, such
animals can be bred with the aforementioned tumor-prone animals to generate a
transgenic animal containing (1) a pair of Lox sites flanking one or both alleles of the
candidate gene, (2) a Cre coding sequence under the control of an inducible promoter,
and (3) a genetic mutation rendering the animal tumor-prone. Cre expression is then
20 induced to knockout the candidate gene ubiquitously. One can not only observe the
effects of the knockout on a tumor arising *de novo* in the animal, but can also
concomitantly observe any undesired effects of the knockout on normal tissues of the
animal. These undesired effects will be indicative of the toxicity of a therapy that
inhibits the expression or activity of the candidate gene.

25 Instead of knocking out the candidate gene, one can also knock down
the expression of the gene using RNA interference. For example, one can generate an
animal transgenic for a GPC-RNAi sequence whose expression is inducible. This
transgenic animal will allow toxicity studies of reducing the expression or activity of
the candidate gene in an otherwise healthy animal.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can

5 also be used in the practice or testing of the present invention. All publications and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting. Throughout this specification and claims, the word "comprise," or variations such as

10 "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Abstract

Methods of validating tumor maintenance functions of a candidate gene in a *de novo* cancer model and methods of establishing therapeutic windows of therapies targeting this gene.

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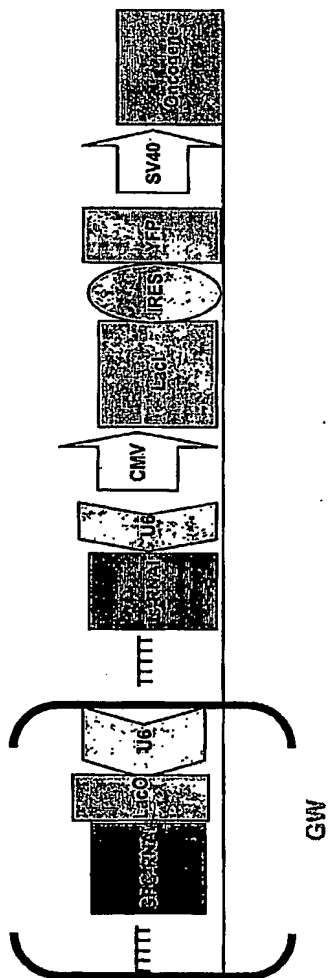


FIG. 1

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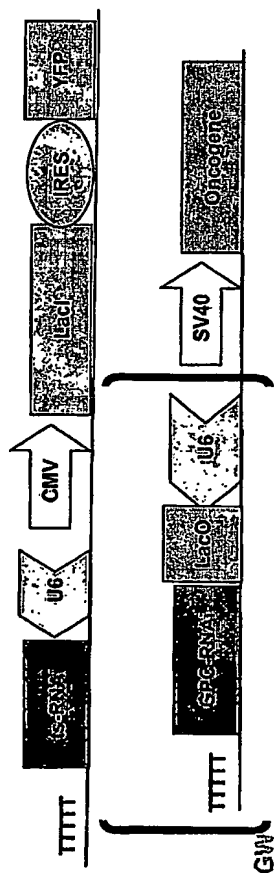


FIG. 2

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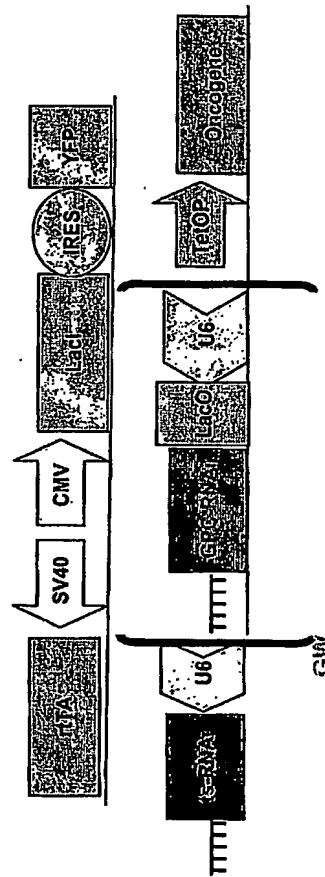


FIG. 3

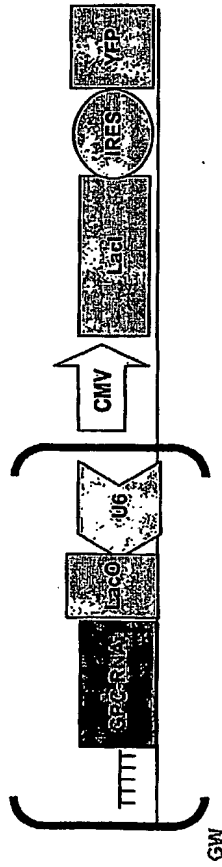


FIG. 4